Research Article

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Thermal inactivation kinetics of polyphenol oxidase in Tea (Camellia sinensis)

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ABSTRACT: Green teas are rich in catechins. Catechins are prone to oxidation. The enzyme polyphenol oxidase present in tea leaves mediates the oxidation of the catechins. Therefore, inactivation of polyphenol oxidase (PPO) is one of the first steps in green tea manufacture. This is primarily done by heating the freshly harvested tea leaves and the process step is commonly referred to as 'fixing' in the tea industry. Various kinetic models for thermal inactivation of PPO were assessed. These included the first order model, fractional model, series model and the two fraction model. The "two-fraction" model was found to explain the thermal inactivation kinetics data of PPO (R^2>0.99) in the tea leaves. Kinetic parameters corresponding to both the heat-labile and the heat-resistant fractions of the enzyme were established from the model. It was found that variation in rate constants with temperature was not governed by Arrhenius relationship.

KEYWORDS: *Camellia sinensis*; Polyphenol Oxidase; Thermal inactivation Kinetics; Mathematical modeling

Introduction

Tea is the most popular non alcoholic beverage in the world. Freshly harvested tea leaves are processed differentially to convert them into green (non-fermented), oolong (semi-fermented) and black (fermented) teas.[11] Polyphenol oxidase (PPO) has been reported to be the governing oxidative enzyme during the fermentation. Green tea processing involves an enzyme inactivation step wherein, enzyme mediated oxidation of polyphenols is inhibited by application of heat.[22]

There is abundant open literature on thermal inactivation of PPO in case of fruits and vegetables like palmito,[13] spearmint,[17] allium,[1] melon[5] and radish.[10] Because of its importance in the food industry, much research has been devoted to thermal inactivation of PPO (Ludhikhuyze et al., 2003).[7,15]

Thermal inactivation of PPO mostly follows first-order kinetics, occasionally 'biphasic' or *n*th-order inactivation[13,10] behaviour has also been observed. 'Biphasic' inactivation kinetics has been ascribed either to the presence of isoezymes that behave differently when subjected to high temperatures[13] or to a two step inactivation process.[21]

In this study, the isothermal curves for the activity of PPO as a function of time was determined at various temperatures and fitted to various enzyme inactivation models available in literature. Mathematical modeling was applied to better explain, interpret and apply the kinetic information obtained from experimentation. Statistical and physical criteria were used to determine the suitability of a model in explaining the thermal inactivation kinetics.

Materials and Methods

(+)-Catechin, polyvinylpyrrolidone (PVPP), benzamidine hydrochloride, phenyl methylsulfonyl fluoride (PMSF), triton X-100, 2-(n-morpholino) ethanesulfonic acid (MES), bovine serum albumin (BSA) were obtained from Sigma Chemical Co. (St. Louis, USA). Other reagents and chemicals used were of analytical grade.

Plant Material

Tea leaves used for this study were obtained from Tea Plantations located in Nilgiris, South India. Tea shoots comprising bud and subtending three leaves were harvested from plots under regular plucking standards. Leaves were bought to lab and stored in deep freezer (-80°C) until they were used for extraction of PPO.

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Preparation of Enzyme Extract

Crude enzyme extract was prepared by the method described by Subramanian *et al.*[23]

Enzyme Assay

Polyphenol oxidase activity was determined by the method of Moore & Flurkey.[16] The reactions were arrested by addition as described by Finger *et al.*[9] One unit of enzyme activity is defined as change in 0.001 absorbance unit at 400 nm per minute. Residual enzyme activity at any given time is the ratio between the enzyme activity at any given time to the enzyme activity at time zero.

Protein Determination

Protein concentration of the various extracts and solutions was determined by the dye-binding method described by Bradford.[3]

Thermal Inactivation Experiments

Thermal inactivation of crude enzyme extract was carried out at different temperatures as well as for different time periods. The temperatures used for the study were 60°C, 70°C, 80°C and 90°C. After heat treatment, enzyme aliquot was immediately frozen in liquid nitrogen to stop any further biochemical reaction. Enzyme extracts were analyzed immediately for residual PPO activity.

Mathematical Modeling

The isothermal inactivation kinetics data of PPO activity at various temperatures was fitted to the following mathematical models available in the literature for thermal inactivation of enzymes:

1. First order model
2. Fractional conversion model
3. Series model
4. Two-fraction model

The first order model assumes that the native form of the enzyme gets inactivated in a single irreversible step. The first order inactivation model has been extensively discussed in literature for various food systems.[2,14,18] The kinetic equation for the first order kinetic model is given by

$$A = A0 \exp(-kt) \quad (1)$$

A: enzyme activity at time t

A0: enzyme activity at time t = 0

The fractional conversion model i s similar in principle to the first order inactivation model. However, the fractional conversion model also takes into account the extremely heat resistant enzyme fraction which does not get inactivated even at time t = ∞. The kinetic equation for fractional conversion model is given by

$$A = A_r + (A_0 - A_r)\exp(-kt) \quad (2)$$

A_r = activity of an extremely heat resistant fraction

k = first order reaction rate constant min^{-1}

The details of fractional conversion model have been described by Rizvi *et al.*[20]

The series model is based on the assumption that the native enzyme (E) goes to its inactive form (E_2) through an intermediate form (E_1) whose activity is less than the native enzyme (E). The Series model can be represented as:

$$E \xrightarrow{k_1} E_1 \xrightarrow{k_2} E_2$$

k_1 = first order reaction rate constant for conversion of native form of enzyme E to the intermediate form E_1 (min^{-1})

k_2 = first order reaction rate constant for conversion of intermediate form E_1 to the in active form E_2 (min^{-1})

The kinetic equation of series model is given by

$$\frac{A}{A_0} = \alpha_2 + [1 + \frac{\alpha_1 k_1}{k_2 - k_1} - \frac{\alpha_2 k_2}{k_2 - k_1}]\exp(-k_1 t) - [\frac{\alpha_1 k_1}{k_2 - k_1} - \frac{\alpha_2 k_2}{k_2 - k_1}]\exp(-k_2 t) \quad (3)$$

α_1 Activity of intermediate form E_1/Activity of native form E,

α_2 Activity of inactive form E_2/Activity of native form E

Series model has been reported by Henley & Sadana.[12]

The two-fraction model is based on the assumption that there exists the presence of multiple isoenzymes which differ in their thermal stabilities.[4] The two fractions have been termed as heat-labile and heat-resistant fractions depending on their stability towards heat. Both the fractions would follow first order inactivation process. The kinetic equation for the two-fraction model can be given by

$$\frac{A}{A_0} = a\exp(-k_l t) + (1-a)\exp(-k_r t) \quad (4)$$

A = fraction of the heat labile isoenzyme

k_1 = first order inactivation rate constant corresponding to the heat-labile fraction (min^{-1})

k_r = first order inactivation rate constant corresponding to the heat-resistant fraction (min^{-1})

The coefficient of determination (R^2) coefficient of correlation and standard error (SE) were the key determinants in assuring the suitability of a model. Low values of (R^2) and high values of (SE) suggested that the model did not explain the thermal inactivation kinetics. The acceptance of a model was also dependent on its physical significance. Models wherein, the kinetic parameters were found negative were rejected.

The dependence of inactivation rate constants on temperature was determined by using Arrhenius relationship and is given by

$$\ln k = \ln k_o - \frac{E_a}{RT} \quad (5)$$

k_0 = pre exponential factor
R = universal gas constant (kcal/mol/K)
T = absolute temperature (K)

Results and Discussion

A step wise procedure was used to evaluate the applicability of models to the experimental data. The isothermal curves for the thermal inactivation of PPO in tea were determined at temperatures ranging from 45°C to 90°C as described in Fig. 1. Higher residual enzyme activity for longer period of time was observed at 45°C compared to other temperatures ranging between 60°C to 90°C. This observation also led to exclusion of kinetics studies at 45°C and hence kinetics data have been discussed for the temperatures ranging between 60°C to 90°C.The thermal inactivation kinetics of PPO in tea was represented by a first order model as shown in Fig. 2. Inactivation of PPO at 45°C also followed a first order reaction (data not included in Fig. 2). However the kinetics data obtained for the temperatures between 60°C to 90°C did not follow the first order reaction pattern as indicated by low R^2 and high standard error (SE) values (Table 1).

The fractional conversion model was subsequently considered. Inactivation of PPO in tea at different temperatures as represented by the fractional conversion model is depicted in Fig. 3. According to this model, there is requirement of residual enzyme activity even at time $t = \infty$ at all temperatures. However, the experimental data does not support the simulated fractional conversion model as no residual enzyme activity was recorded at any of the temperatures. Thus, the fractional conversion model was unable to explain the thermal inactivation kinetics data. This is further substantiated by high SE values (Table 2). Hence, the presence of an extremely heat resistant enzyme fraction was ruled out. The values of k and A_r at different temperatures were calculated and are also shown in Table 2.

The data was then applied on the series model as shown in Fig. 4. In case of series- type model, incorrect parameter estimates (i.e. negative values of α_2) were obtained

(Table 3). The specific activity of initial enzyme state α_1 was less than 1, and the final state was almost deactivated ($\alpha_{12} \sim 0$; Table 3). There was no evidence in the experimental data for the presence of intermediate form of enzyme and, therefore this model was not accepted.

The fourth model that was assessed was the two-fraction model. PPO inactivation in tea as represented

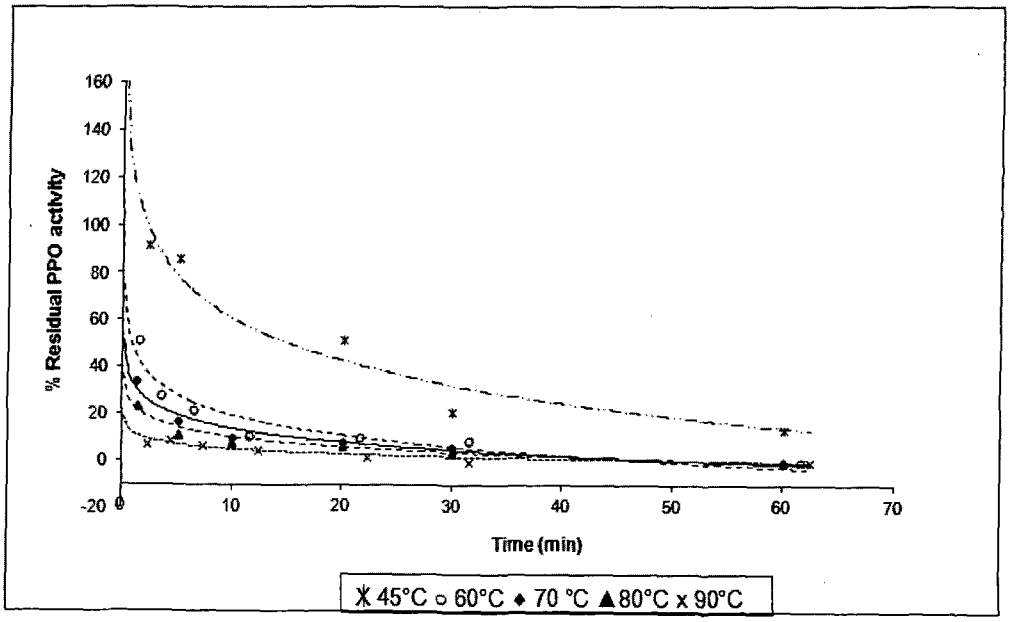

Fig. 1: Residual activity of PPO.

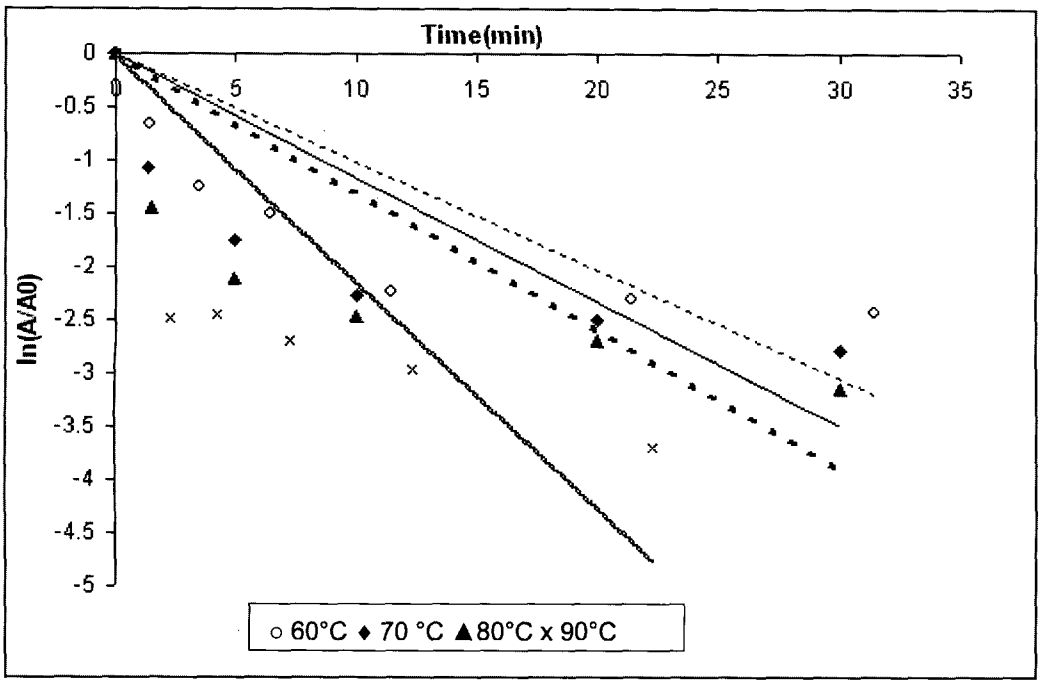

Fig. 2: First order model.

Table 1: Computed values of kinetic parameters for first order model for PPO in activation in tea

Temperature (° C)	k (min^{-1})	R^2	SE
60	0.0306	0.2925	1.003
70	0.1200	0.2660	1.164
80	0.1300	0.1146	1.2573
90	0.2134	Found negative	1.4022

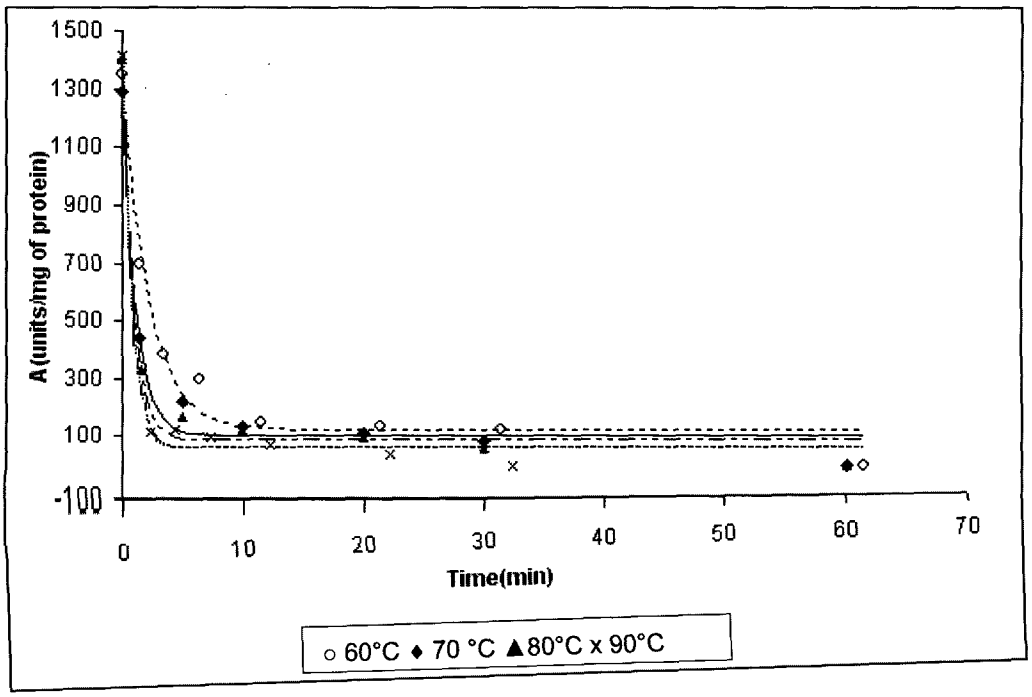

Fig. 3 : Fractional conversion model.

Table 2: Computed values of kinetic parameters for fractional conversion model for PPO inactivation in tea

Temperature (° C)	k (min^{-1})	A_r (units/ mg of protein)	R^2	SE
60	0.4533	119.4331	0.9767	478.3283
70	0.9000	103.3300	0.9805	492.3059
80	1.1100	87.0492	0.9892	538.3347
90	1.3300	62.8956	0.9941	557.3436

Table 3: Computed values of kinetic parameters for Series model for PPO inactivation in tea

Temperature (° C)	$k_1(min^{-1})$	$k_2(min^{-1})$	α_1	α_2	R^2	SE
60	0.6481	0.0395	0.2260	-0.0053	0.9954	0.3543
70	0.9111	0.9084	0.0090	0.0064	0.9391	0.3807
80	1.4296	0.04124	0.1446	-0.0040	0.9996	0.3845
90	4.0831	0.0625	0.1080	-0.0037	0.9996	0.3666

Table 4: Computed values of kinetic parameters for two fraction model for PPO inactivation in tea

Temperature (° C)	a	k_l (min^{-1})	k_r (min^{-1})	R^2	SE
60	0.7598	0.6579	0.0423	0.9953	0.3543
70	0.7999	1.2134	0.0472	0.9987	0.3807
80	0.8533	1.4395	0.0440	1.000	0.3807
90	0.8927	6.7305	0.0677	0.9996	0.3666

by the two-fraction model is shown in Fig. 5. The values of R^2 are high and values of SE are low. The model satisfies the physical criteria of consideration since none of the kinetic parameters a, k_l and k_r were found to be negative at any temperature. Thus, amongst all the models evaluated, the two-fraction model possibly best explains the thermal inactivation kinetics of PPO in tea leaves. This finding also corroborates the presence of multiple isoenzymes of PPO in tea well as in other fruits as suggested by some previous studies.[6,10,23] Computed values of these kinetic parameters at different temperatures are shown in Table 4. Tea PPO biphasic inactivation profile seems similar to the inactivation kinetics reported in the other fruits/vegetable like palmito, spearmint, radish etc summarized in Table 5.

Linear regression using equation 5 was carried out to give dependence of inactivation rate constants obtained from two-fraction model on temperature. The plots of ln k_l vs. 1/T and ln k_r vs. 1/T did not give the best fits (R^2=0.8569 and 0.6309, respectively). Thus, the variation of rate constants with temperature was not governed by Arrhenius relationship.

This finding is in accordance with the results of some previously reported studies.[8,10,19]

Conclusion

Thermal inactivation of PPO does not obey simple first order kinetics. Data modelling studies indicated that thermal inactivation of PPO in tea occurs in two phases with the first phase perhaps corresponding to the inactivation of the heat labile form and the other

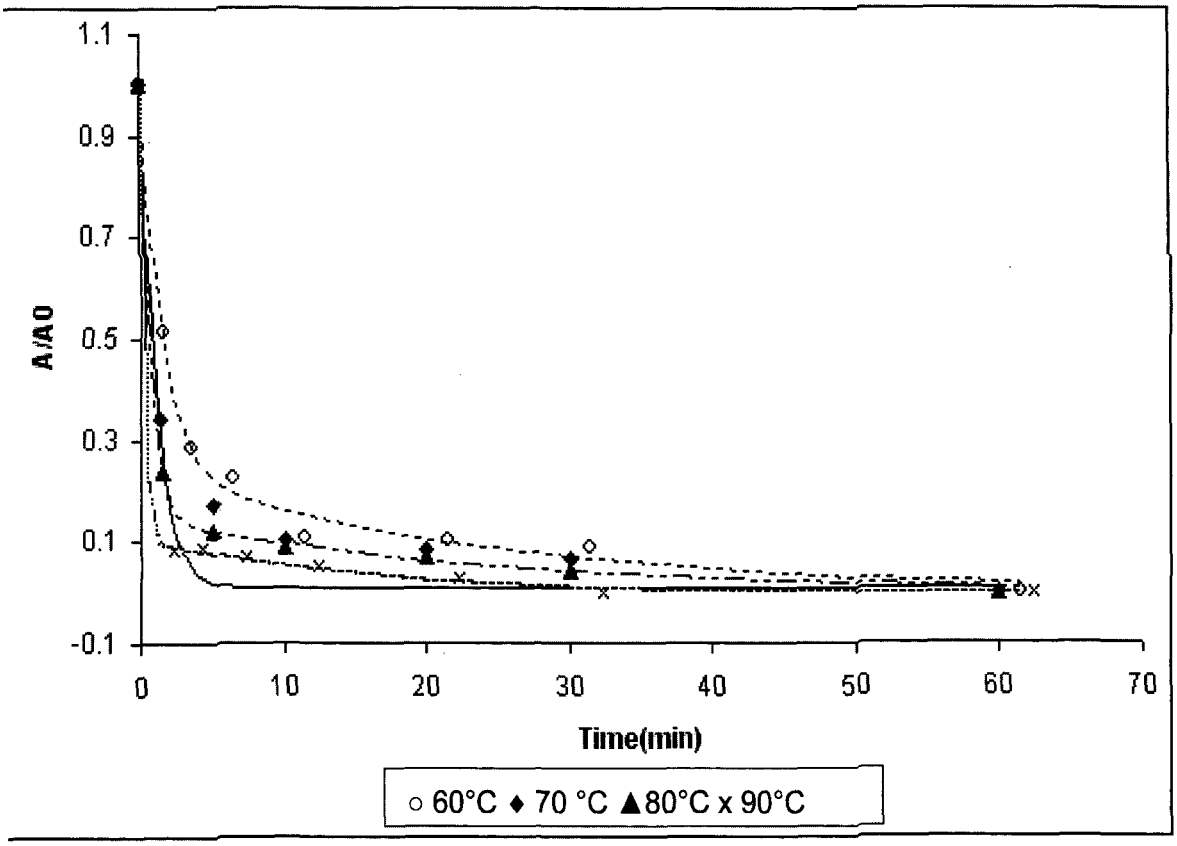

Fig. 4 : Series model.

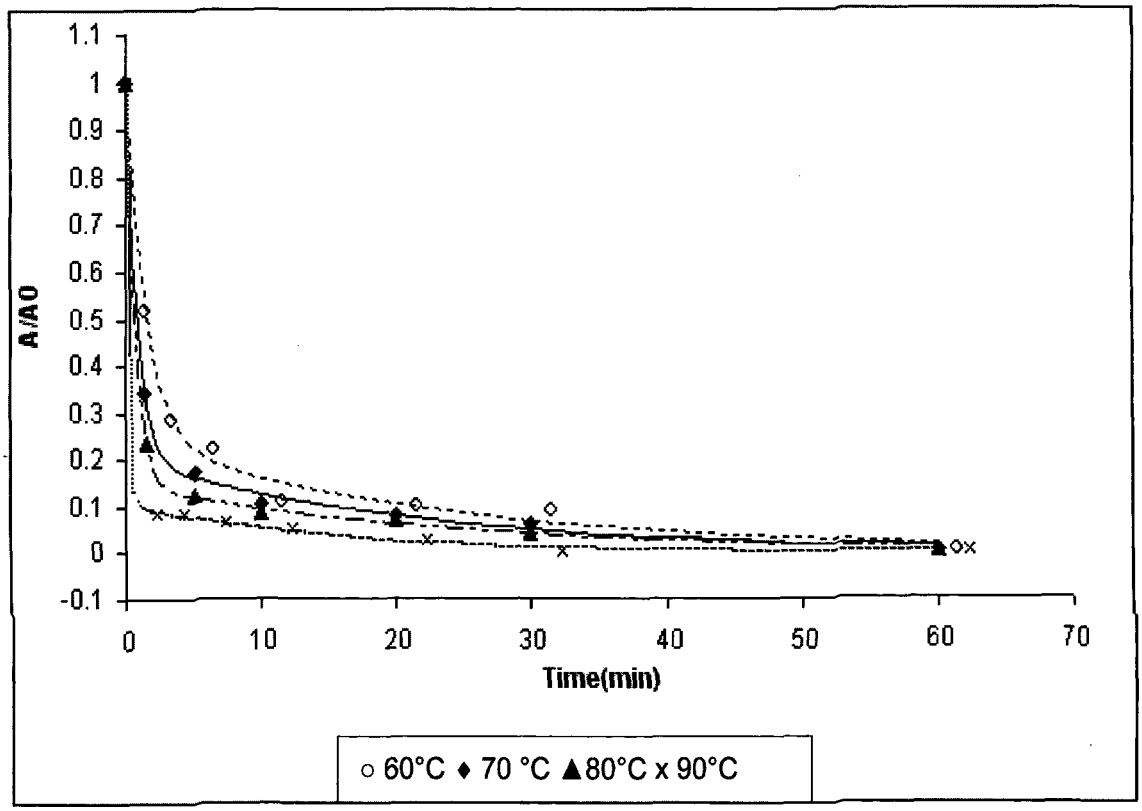

Fig. 5 : Two-fraction model.

Table 5: Comparison of PPO inactivation kinetics in other fruits/vegetables

Vegetable	Reference	Findings of study	Comparison with tea PPO
Palmito	Lourenço et al., 1990	Inactivation is biphasic and there is a presence of multiple isoenzymes of varying heat stability	At T =70° C and t =3 minutes % residual activity = 60% (palmito) % residual activity = 20% (tea) At T=70° C and t =25 minutes % residual activity = 20% (palmito) % residual activity = 10% (tea)
Allium sp.	Arslan et al., 1997	Inactivation is biphasic	At T=60° C and t =3 minutes % residual activity = 90% (Allium sp.) % residual activity = 30% (tea) At T=80 ° C and t =3 minutes % residual activity = 70% (Allium sp.) % residual activity = 10% (tea)
Spearmint	Neves et al., 2009	Inactivation is biphasic	At T=70° C and t =3 minutes % residual activity = 45% (spearmint) % residual activity = 20% (tea)
White yam	Eze et al., 2010	Inactivation is biphasic	At T=60° C and t =3 minutes % residual activity = 95% (Dioscorea rotundata) % residual activity = 30% (tea)
Radish	Goyeneche et al., 2013	Inactivation is biphasic	At T=60° C and t =3 minutes % residual activity = 45% (Raphanus sativus) % residual activity = 30% (tea)

to that of the heat resistant form. Amongst the models selected for study, the two-fraction model was found to be the best suited model (R^2 >0.99) for tea. However, to unravel the mechanism of thermal inactivation of PPO, experimental studies with each isoform needs to be evaluated.

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